MS has made a number of important contributions to drug metabolism and the further development of the technique, together with advances in instrumentation, will enhance its application in this area.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry. **Chromatography: Liquid:** Detectors: Ultraviolet and visible Detection; Nuclear Magnetic Resonance Detectors. **Chromatography: Thin-Layer (planar):** Layers. **Electrophoresis:** Capillary Electrophoresis; Capillary Electrophoresis Detection; Capillary Electrophoresis-Mass Spectrometry; Capillary Electrophoresis-Nuclear Magnetic Resonance. **Extraction:** Analytical Extractions; Solid-Phase Extraction; Solvent Based Separation; **III/Drugs of Abuse: Solid-Phase Extraction. Drugs and Metabolites:** Liquid Chromatography-Mass Spectrometry; Liquid Chromatography-Nuclear Magnetic Resonance-Mass Spectrometry.

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ESSENTIAL GUIDES FOR ISOLATION/ PURIFICATION OF ENZYMES AND PROTEINS

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Nature of the Problem

The purification of proteins presents a unique challenge in the field of separation science. Typically, the particular protein to be isolated will constitute 1% or less (sometimes much less) of the material in the original extract and all of the contaminants will have basically the same chemical characteristics, i.e. they are all proteins. There is the added complication that for most purposes it is necessary to retain the biological activity of the protein, and the inherent instability of protein structures restricts the range of temperatures and solvent compositions that can be used during purification.

Tools for its Solution

Clearly, methods for the separation of proteins must be based on those characteristics in which they differ from one another. The most important of these are listed in **Table 1** along with the separation techniques that exploit those differences. These various properties are not of equal generality or of equal utility for purification purposes.

By far the most widely used technique for protein isolation is ion exchange chromatography. The generality of the method arises from the fact that proteins contain ionizable amino acids and hence carry a net charge at all pH values except the unique pH (the isoelectric point) at which the positive and negative charges are equal. Moreover, two proteins that have the same charge at a particular pH are likely to differ in charge at some other pH. Ion exchange chromatography is technically simple and can be adopted for use over a very large range of scales. Chromatofocusing and isoelectric focusing are methods that depend on the differences in isoelectric points between proteins but are less widely used for preparative work than is ion exchange chromatography because of increased cost, restrictions of scale and technical difficulty.

Electrophoresis is a special case. Electrophoretic methods are of central importance in analytical protein chemistry but, until recently, have not proved

Table 1 Properties of proteins that can be exploited for purification and associated experimental methods

Electrical charge	Ion exchange chromatography Chromatofocusing Isoelectric focusing (Electrophoresis)
Hydrophobic surface regions	Hydrophobic chromatography
General surface properties	Salt fractionation
Size	Size-exclusion chromatography Membrane filtration
Specific binding site	Affinity chromatography Dye-binding chromatography
Surface carbohydrate	Lectin chromatography
Metal-binding site	Metal chelate chromatography
Antigenic determinants	Immunoaffinity chromatography

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useful for purification purposes. The reason for the change has been the development of ultra high sensitivity techniques for structural analysis that has blurred the distinction between analytical and preparative methods. Hence the inclusion of electrophoresis in Table 1 as a preparative method, although it serves that purpose for a limited range of applications (see later).

Although the surfaces of most soluble proteins are predominantly polar, many of them have patches of hydrophobic amino acids that, under appropriate conditions (usually at high salt concentrations), can bind to hydrophobic matrices. This provides a method for separation provided that elution from the matrix can be achieved under conditions that do not lead to loss of biological activity.

Proteins differ from one another in their solubilities in salt solutions. Clearly, in a complex mixture of proteins the solubilities of the components will overlap and hence fractional precipitation with salt, usually ammonium sulfate, provides only a crude separation method. However, it is widely used as a first step in purification procedures, particularly when working on a large scale. Occasionally, fractional precipitation with an organic solvent (ethanol, acetone) is used but there is a possibility of protein denaturation at high solvent concentrations.

Proteins also differ from one another in size and this can be exploited in size-exclusion chromatography. This is inherently a method of low resolution and can rarely achieve more than separation of mixtures of proteins into broad size classes. However, a very important application of size-exclusion chromatography is for changing the composition (e.g. the pH) of the solvent between steps in a purification procedure. Dialysis can also be used for this purpose but is much slower. The same restriction of low resolution applies to separations using membrane filtration, but this technique is of enormous utility at various stages in a purification schedule for reducing the volume of protein solutions: the single major 'contaminant' in a protein solution is water.

Whereas the methods above depend on differences in structures of proteins there is also a set of procedures that depend essentially on differences in biological activity. In the vast majority of cases, biological activity of a protein depends on it recognizing and binding to a ligand. For example, enzymes bind to substrates and inhibitors, hormones bind to receptors, antibodies bind to antigens and so on. This specific biological activity can be exploited by construction of a matrix to which the appropriate ligand is (usually covalently) attached. Passage of a protein mixture through the resulting affinity matrix should result in binding of one or a small number of proteins that recognize the ligand. Subsequent elution can be achieved by passage of a solution of the ligand, or a suitable analogue, through the column. This method has seen widespread application in the purification of enzymes and is in principle capable of very high selectivity because of the specificity of enzyme/substrate or enzyme/inhibitor binding. The selectivity achieved is, however, often limited by the fact that the ligand may be charged and hence gives rise to ion exchange effects, or it may be hydrophobic and give rise to nonspecific hydrophobic interactions. Despite this, affinity chromatography is a very powerful method and its use is restricted more by the fact that it is often necessary to design and synthesize the affinity matrix oneself rather than by inherent limitations.

Dye binding chromatography is a variant of affinity chromatography and relies on the fact that a variety of chlorotriazine textile dyes interact moderately specifically with enzymes that have nucleotide (ATP, NAD(H), coenzyme A (CoA)) binding sites. The ability of dye-containing matrices to recognize nucleotide-dependent enzymes is not a purely affinity effect – indeed the structural similarity between the dyes used and the cofactors is not obvious – and includes elements of ion exchange and hydrophobic effects. Nevertheless, these methods often work remarkably well for isolation of groups of nucleotide-dependent enzymes or even of individual members when biospecific elution methods are used.

Lectin chromatography and metal chelate chromatography are available when the protein of interest has either surface carbohydrate or a metal-binding site, respectively. The former method depends on the fact that various plants produce proteins (lectins) that bind specifically to particular classes of carbohydrate. If the lectin is coupled to an appropriate support then the product matrix will specifically bind glycolproteins from a mixture of proteins. Elution can be effected by passage of a solution of the appropriate monosaccharide through the column. In metal chelate chromatography the matrix has a chelating agent covalently attached and loaded with an appropriate metal ion. On passage of a mixture of proteins through the column those with a binding site for the metal will be retained and subsequently can be eluted by passage of a solution of metal ions through the column.

Immunoaffinity chromatography is in principle the most specific method available for protein isolation. It involves raising an antibody to the target protein, attaching the antibody to a supporting material and then using this as an affinity matrix. The extreme specificity of antigen–antibody interactions should ensure high selectivity in binding the

target protein. However, there are two problems. Clearly, the protein has to have been isolated previously in order for an antibody to be produced. A highly purified protein will be required to raise polyclonal antibodies. Alternatively, a partly purified antigen can be used to produce monoclonal antibodies but this adds an extensive new dimension to a purification procedure. The major restriction on the application of the method, however, is the difficulty of elution of proteins from the immunoaffinity matrix once bound. The tightness of binding often requires extreme conditions for efficient elution (very high or low pH, presence of chaotropic agents) such that many protein molecules become denatured during the elution process.

Putting them Together

Faced with the variety of methods available for the separation of proteins the question arises as to which of them to use and in which order for development of a purification schedule for a particular protein. The answer to this depends on a whole host of issues such as:

- how much protein is required?
- what sources of the protein are available?
- has the gene for the protein been cloned?
- is the protein required to be completely pure?
- is it necessary to retain biological activity?
- has it been done before?

If the answer to the last question is positive, the obvious approach is to try to reproduce the reported purification procedure. It may not work exactly as described – small variations in procedures between laboratories can give rise to significant differences in the behaviour of proteins during purification – but it should be relatively easy to adjust conditions to get it right. Development of a new protocol is time-consuming and not usually worthwhile unless it is to be used repeatedly and an existing method appears to be unnecessarily cumbersome; even then the published method should provide a valuable guide on how to make improvements.

What follows are descriptions of the sorts of schedules of methods that might be used in a variety of situations.

Large-Scale Isolation of an Active Protein

Large-scale here is taken to mean a laboratory-scale purification of a few tens of milligrams of protein. Industrial scale purification might well follow the same general pattern but there would be engineering problems associated with scaling up that will not be dealt with here. In the case of a protein to be used for therapeutic purposes there would also be specific requirements imposed by regulatory agencies that are beyond the scope of the present discussion.

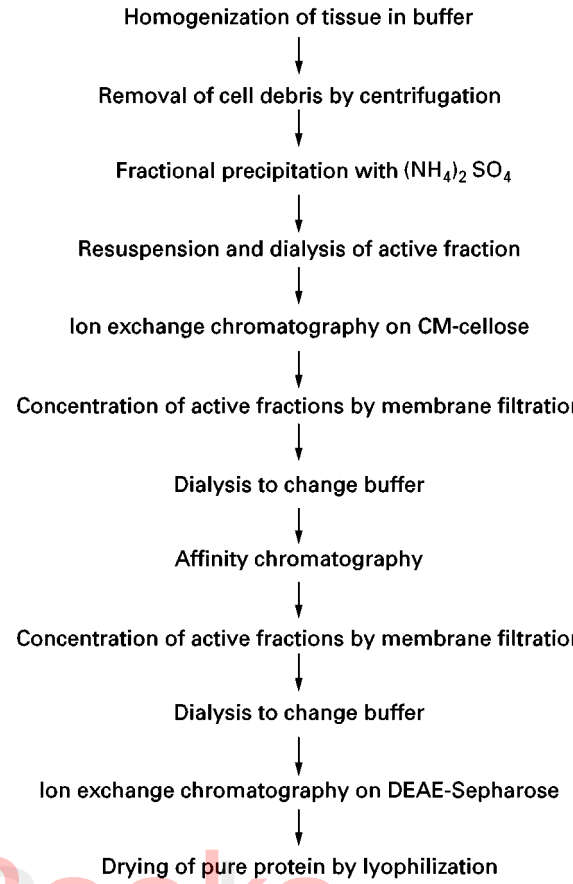
Homogenization of tissue in buffer
Removal of cell debris by centrifugation
Fractional precipitation with $(NH_4)_2 SO_4$
Resuspension and dialysis of active fraction
Ion exchange chromatography on CM-cellose
Concentration of active fractions by membrane filtration
Dialysis to change buffer
Affinity chromatography
Concentration of active fractions by membrane filtration
Dialysis to change buffer
Ion exchange chromatography on DEAE-Sepharose
Drying of pure protein by lyophilization

Figure 1 Flow chart for the purification of an enzyme.

The flow chart in **Figure 1** outlines steps in the protocol for purification of an enzyme developed in the author's laboratory. The starting material was 5 kg of pig liver. If the source of the enzyme or other protein is not of importance for the purpose of the investigation then the best choice is to use an animal tissue that can be obtained in quantity from a commercial abattoir. Animal tissues are generally easy to homogenize in a domestic food blender. Other sources such as fungi, bacteria and plants present difficulties in disruption of the tissue and are best avoided unless the source is constrained by the problem in hand.

Ten litres of buffer was used for homogenization and, after removal of debris, the volume of protein solution was 8.5 L. This volume of solution is difficult to handle and hence fractional precipitation with ammonium sulfate was used both to obtain an initial crude purification and, more importantly,

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reduce the volume. After centrifugation of the active fraction, resuspension of the pellet and dialysis the volume was reduced to 500 mL.

The next step was ion exchange chromatography. There were choices to be made of whether to use a cation or anion exchanger, and as to which of the various available supporting materials (cellulose, Sepharose, Superose) was to be preferred. In the present case, a cellulose-based matrix was chosen. This was essentially because the amount of protein in the sample (about 100 g) made it necessary to use a large amount of exchanger and correspondingly a large column. Cellulose-based exchangers are much cheaper than other varieties and, in addition, large columns of cellulose exchangers have better flow characteristics than do those of other materials. The choice of carboxymethyl (CM) cellulose rather than the anion exchanger diethylaminoethyl (DEAE) cellulose was dictated by previous experience of the behaviour of the two materials for the separation of crude protein mixtures.

The protein of interest was retained by the CM-cellulose and was eluted by application of a gradient of increasing sodium chloride. This is to be preferred over the other possibility of using conditions where the protein is not retained on the column since a higher degree of purification is likely to be achieved on gradient elution. After combination and concentration of the active fractions the volume of the sample had been reduced to about 50 mL and the amount of protein to about 1 g. These amounts were suitable for the application of a variety of small-scale but more highly resolving techniques. For example, had the enzyme of interest been a glycoprotein then lectin affinity chromatography would have been a good choice. Similarly, hydrophobic chromatography could have been used. An advantage of using the latter technique would have been that it would not have been necessary to remove the sodium chloride from the sample after gradient elution from CM-cellulose given that in hydrophobic chromatography the sample is applied in a solution of high salt content to promote interaction with the matrix.

In practise it was relatively easy in the present case to develop an affinity matrix for the enzyme based an analogue of the substrate. It was worthwhile to do this because it was intended to repeat the purification frequently so that the time involved in preparing the affinity matrix was subsequently recovered. If a purification is essentially one-off then this is unlikely to be the case.

After affinity chromatography the product was examined by electrophoresis and found to contain two minor contaminants, both more basic than the target enzyme. Hence, a final step using an anion exchanger under conditions where the protein of interest was absorbed but the contaminants were not, or were bound more weakly, suggested itself. The exchanger chosen was DEAE-Sepharose, which has a greater resolving power than cellulose-based products.

The final product of the purification procedure was 28 mg of protein that was homogeneous, as judged by the usual criterion of producing a single band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme had been purified 6000-fold compared with the original homogenate and the yield was about 50%.

Small-Scale Isolation of an Active Protein

Isolation of a few milligrams of active protein follows the same general principles as outlined above but can often be achieved in a smaller number of steps. For example it is not necessary to carry out fractional precipitation with salt because the small volume of protein solution will allow direct use of ion exchange chromatography as the first purification step. In addition, because of the small scale of the procedure, the high resolving power of ion exchange chromatography or of hydrophobic chromatography in fast protein liquid chromatography (FPLC) mode can be exploited, which may allow a reduction in the number of chromatiographic steps required. FPLC differs from conventional column chromatography in that it employs very fine particle size matrices that offer greater resolving power for protein mixtures. Fully automated equipment is also available that allows for greater reproducibility between runs than do conventional methods. Capacity is, however, limited.

Proteins from Sub-Cellular Organelles

Many proteins in higher organisms exist in discrete subcellular organelles such as mitochondria, chloroplasts and lysosomes. If the target protein is one of these it may be advantageous to make a preparation of the organelle and isolate the protein from that rather than from a total issue homogenate. Isolation of subcellular organelles is usually carried out by preparative differential centrifugation. Proteins can subsequently be extracted from the organelles and purified by standard techniques.

The advantage of this approach is obvious. Given that the organelles contain a more restricted range of proteins than does the parent cell then the purification procedure is likely to be much simpler. The downside is that the isolation of subcellular structures is time-consuming and, except on a relatively small

scale, there may not be a net saving of time in adopting this approach.

Membrane Proteins

Integral membrane proteins present special problems because of their location within membranes and because they are not soluble in aqueous buffer solutions. The first step will be to obtain a preparation of the membrane of interest, usually by differential centrifugation. Next, the protein has to be extracted from the membrane preparation, most commonly by using solutions of detergents such as Triton X-100, Lubrol PX, digitonin, sodium cholate, etc. This is a crucial step and the best detergent to use to obtain optimum release of the protein from the membrane fragments can be determined only by trial and error.

Once a soluble extract of the protein has been obtained its purification can be achieved using the usual chromatographic techniques except that, because of solubility problems, it will be necessary to maintain a standing concentration of detergent in the buffers. This frequently adversely affects the performance of ion exchange materials and more success in isolation of membrane proteins has been achieved by exploiting their binding properties, that is, by using various forms of affinity chromatography.

A final problem, once the protein has been purified, will usually be to remove the detergent from the preparation or to change the detergent type. This can be achieved by a variety of methods, including equilibrium dialysis, gel filtration and a variety of chromatographic methods.

Peripheral membrane proteins, that is, those that are only loosely associated with the membrane, do not usually present special problems. They can be released from membrane preparations by salt extraction or by changes in pH, are usually soluble in aqueous buffers, and are amenable to the usual purification methods.

Products from Cloned Genes

As a result of the rapid developments in genetic technology in recent years it is now relatively easy to clone the gene for any protein of interest and express it in a suitable bacterial host. This does not change the methods that are available for purification but it does allows for simplification of the purification procedure. An obvious example is that expression of the gene can be manipulated so that its protein product represents a very high percentage of the protein in the host cell. Values of up to 50% have been achieved, which obviously simplifies the subsequent purification. Similarly, some success has been achieved in modifying genes by the attachment of an export signal so that the host organism excretes the protein product into the culture medium.

Other approaches to facilitating purification of cloned gene products involve the construction of fusion proteins. One example of this is where a tail of basic residues (lysine or arginine) is engineered onto the protein. This tail will make the protein very basic and hence increase its affinity for ion exchangers such as CM-Sephadex. If, after elution from the exchanger, further purification is required then the tail can be removed (by digestion with carboxypeptidase B) followed by further chromatography under the same conditions. The decreased basicity consequent on removal of the tail will ensure that the protein now behaves differently compared with any contaminants whose properties will not have been modified.

Other approaches involve engineering affinity labels onto the protein. For example, fusion products between a target protein and maltose-binding protein from *Escherichia coli* can be very readily purified by amylose affinity chromatography. Similarly, antibodies to certain small peptide sequences, referred to as flags, have been raised so that fusion proteins bearing these flag sequences can be readily purified by immunoaffinity chromatography.

Obviously, in any particular case the question needs to be asked as to whether the time and cost involved in genetic engineering of the desired protein product is justified in terms of the time saved in subsequent purification. The answer is likely to be positive only if the purification is to be repeated frequently.

Special Cases

The procedures described above should be used when it is important to retain the biological activity of the protein of interest. Essentially, this means using experimental conditions under which the native three-dimensional structure of the protein is preserved. There are some situations where this is not necessary and all that is important is that the primary structure of the protein remains unchanged. An important example of this is where the protein is required for amino acid sequence analysis. In this case additional techniques can be used for purification. For example reverse-phase HPLC using hydrocarbon (C_4–C_{18}) stationary phases provides for high-resolution separation of proteins but elution often requires the use of organic solvents such as acetonitrile, which frequently leads to denaturation. The method is, however, extremely powerful for final

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separation of partly purified proteins for sequence analysis.

For a variety of applications, including *N*-terminal sequence analysis using modern high-sensitivity techniques, only very small amounts of protein (a few micrograms) are required. For these applications the resolving power of SDS-PAGE can be exploited to separate even relatively crude mixtures. The protein of interest is then removed from the acrylamide gel, for example by using an appropriate blotting technique, and the blot subjected to analysis.

More recently this approach has been extended to the identification of proteins in cell homogenates. The total cell extract is separated by two-dimensional electrophoresis, most commonly using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. Individual spots are excised from the gel, the protein subjected to digestion with trypsin, and the trypsin fragments analysed by mass spectrometry. The set of peptide masses obtained is then scanned against a data bank of the masses of tryptic peptides from all known proteins. In most cases this allows unique identification of the protein in the gel spot, provided that its sequence is known either from direct analysis or by translation of a DNA sequence.

Detection and Quantification

It is clearly of central importance in any purification procedure that a method is available for detecting the presence of the protein of interest in the fractions from the various separation steps. In the case of enzymes this is easy because they possess catalytic activity that can be measured by some appropriate analytical technique. Other proteins might require the use of a bioassay, or an immunoassay, or perhaps the identification of the protein as a particular band produced on analytical electrophoresis.

What might not be so obvious is the importance of quantification of the recovery of the protein at each stage of the purification procedure – that is, of keeping an inventory. Unless this is done it is very easy to end up with a disappearing yield of the protein of interest and not to know at which step or steps it disappeared. At each step it is important to measure the total protein content and the amount of the protein of interest. This allows not only the recovery but also the degree of enrichment of the protein to be determined. Any step for which either of these is low should be abandoned.

In the case of enzymes, keeping this inventory is straightforward; it is simply necessary to measure the catalytic activity of a known volume of the fractions. In other cases it is much more difficult. Bioassays can be very time consuming. Immunoassays are not usually too difficult, but in this case it is necessary to bear in mind that immunological reactivity of a protein may be retained even though biological activity has been lost. In the case of a protein with no known biological activity, or where the activity is very difficult to measure, then recovery can be assessed from the measurements of the intensity of the appropriate band produced by analytical electrophoresis. Whatever the difficulties, however, keeping a score card is essential if a successful purification protocol is to be developed.

See also: **I/Affinity Separation. Centrifugation. II/Affinity Separation:** Hydrophobic Interaction, Chromatography; Immobilised Boronates and Lectins; Immunoaffinity Chromatography. **Chromatography:** Protein Separation; Size Exclusion Chromatography of Polymers. **Chromatography: Liquid:** Mechanisms: Size Exclusion Chromatography. **Electrophoresis:** Isoelectric Focusing; Two-dimensional Electrophoresis. **Membrane Separations:** Membrane Bioseparations. **III / Proteins:** Centrifugation; Electrophoresis; Field Flow Fractionation; High-Speed Countercurrent Chromatography; Ion Exchange.

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